ACTA ABP BIOCHIMICA POLONICA

Vol. 69, No 3/2022
613–618
https://doi.org/10.18388/abp.2020_5982

Regular paper

Resveratrol inhibits cerebral aneurysms in mice *via* downregulating the NF-κB pathway

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Objectives: Cerebral aneurysm (CA) is one of the most common cerebrovascular diseases. The study was conducted to investigate the effect of resveratrol (RES) on the CA formation and its possible mechanisms. Materials and Methods: Murine model of CA was constructed by induced hypertension and fed without (model group) or with RES (RES group). A Sham group was used as a control. The CA formation and inflammatory response were examined morphologically and histochemically. The expression of nuclear factor-κB (NF-κB), matrix metalloproteinase (MMP)-2, and MMP-9 was analyzed using qRT-PCR and Western blots. Results: CA was induced in mice after the left common carotid artery was ligated and fed with high sodium chloride. Compared with the model, mice fed with RES had significantly fewer CA with smaller size, normal thickness of the arterial wall (P<0.05), and fewer infiltrated macrophages in the aneurysm wall (P<0.05). qRT-PCR and Western blot analyses showed that the expression of MMP-2, MMP-9 and NF-κB was significantly elevated in the model as compared with the control and significantly decreased after RES treatments (P<0.05). Conclusions: RES can inhibit the CA formation in mice subjected to induced hypertension and this inhibition is likely mediated *via* downregulating the NF-κB pathway.

Keywords: cerebral aneurysm; cerebrovascular diseases; resveratrol; NF-κB pathway; matrix metalloproteinase; extracellular matrix

Received: 25 October, 2021; revised: 02 December, 2021; accepted: 20 February, 2022; available on-line: 13 September, 2022

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Acknowledgements of Finanacia Support: This study was supported by a grant (2016YFC0901003) from the Ministry of Science and Technology of China

Abbreviations: ANOVA, of variance; CA, cerebral aneurysm; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HE, hematoxylin and eosin; iNOS, induced nitric oxide synthase; IL-1, interleukin-1; LPS, lipopolysaccharides; MMP, matrix metalloproteinase; MAP, mean arterial pressure; NF-κB, nuclear factor-κB; qRT-PCR, real-time quantitative reverse transcription-polymerase chain reaction; RES, resveratrol; SAH, subarachnoid hemorrhage; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; VPR, volume pressure recording; PVDF, Polyvinylidene fluoride

INTRODUCTION

A cerebral aneurysm (CA) or intracranial aneurysm is one of the most common cerebrovascular diseases and the incidence in the general population ranges from 1% to 5% (Vlak *et al*., 2011). The majority of unruptured CA is asymptomatic or presents as common symptoms such as chronic headache (Baron, 2015). Several factors have been shown to be associated with aneurysm formation, growth, and rupture. For instance, women have been shown to have a higher risk of CA, due to estrogen deficiency (Desai *et al*., 2019) the prevalence of asymptomatic aneurysms is high in middle-aged and elderly patients of both sexes (Horikoshi *et al*., 2002). Inadequate intake of dietary antioxidants, hyperhomocysteinemia, hypertension, and alcohol consumption are risk factors for aneurysms (Czekajlo, 2019). Smoking, age, family history, hypertension, and geographical regions also have an impact on aneurysms (Backes *et al*., 2016). Rupture of intracranial aneurysm is the main cause of subarachnoid hemorrhage (SAH), which ranks the third in cerebrovascular diseases, second only to cerebral thrombosis and hypertensive intracerebral hemorrhage, and has a high mortality and disability rate of up to 25–50% (Hop *et al*., 1997). The surviving patients often have various complications such as rebleeding and delayed cerebral ischemia (van Gijn *et al*., 2007). Craniotomy and endovascular interventional therapy are two effective but invasive treatments for CA with varying degrees of surgical risks and limitations (Bowles, 2014). Therefore, it is highly desirable to develop effective and non-invasive treatments for aneurysms (Bowles, 2014; Golledge *et al*., 2009).

It is believed that CA is formed as a result of the pathological reconstruction of the cerebral artery wall after the degradation of the extracellular matrix (ECM) (Bruno *et al*., 1998; Zhang *et al*., 2019). Previous studies indicated that the infiltration of inflammatory cells and elevated matrix metalloproteinase (MMP) activity are engage in the abnormal degradation of ECM (Aoki *et al*., 2007a; Sarrafzadeh *et al*., 2012; Shinde & Frangogiannis, 2014). During the pathological process, nuclear factor-κB (NF-κB) is abnormally activated (Sun *et al*., 2006), leading to the increased inflammatory response in CA lesion and rupture of saccular intracranial aneurysms (Pawlowska *et al*., 2018).

Resveratrol (RES) is a polyphenol compound widely existing in plants such as *Polygonum cuspidatum*, *Cassia tora*, grape, and peanut (Wang *et al*., 2019). It is demonstrated to have many pharmacological activities, such as radical scavenging, antioxidation, and anti-inflammation (de la Lastra & Villegas, 2005; Galiniak *et al*., 2019). RES could down-regulate the expression of NF-κB to suppress the expression of NF-κB-dependent genes, leading to the reduced inflammatory response. Previous, RES was found to suppress the growth of abdominal aortic aneurysm via up-regulating angiotensin-converting enzyme 2 in mice (Moran *et al*., 2017) or *via* attenuation of inflammation, oxidative stress, and neovascularization (Kaneko *et al*., 2011a; Palmieri *et al*., 2011a). However, it is not clear if RES could inhibit the CA formation.

In this study, we aimed to examine the effect of RES on the CA formation and the possible mechanism underlying the effect. The findings would provide clues to developing a new therapeutic approach for CA.

MATERIALS AND METHODS

Animals

Four-week-old, special pathogen-free C57BL/6 female mice, weighing 20 to 25 g, were purchased from the Medical Animal Experiment Center at Nanjing Medical University (permit no. scxk (Su) 2019-0022). Animals were housed in a constant environment (temperature 22±2°C, humidity 50±5%) with a 12 h day/12 h night light cycle. The animals were housed in hygienic and pathogen-free conditions in large collective cages with three mice per cage. The care of mice was taken by well-trained and dedicated technicians. The animal experimental protocols were approved by the Ethics Committee of Animal Experiment Study of Nanjing Medical University.

Reagents and equipment

RES was purchased from Sigma, USA; Trizol kit was purchased from Invitrogen, USA; cDNA synthesis kit was obtained from Takara, Japan; antibodies against NF-κB, MMP-2, MMP-9, and GAPDH were purchased from Abcam, UK; horse-reddish peroxidase-labeled goat anti-mouse IgG secondary antibody was purchased from Cell Signaling Technology, USA; Chemiluminescent Western Blotting Detection kit was purchased from Thermo Fisher Scientific, USA; Noninvasive CODA tail-cuff monitor was purchased from Kent, USA; quantitate PCR instrument (ABI 7500) was purchased from Applied Biosystems, USA; gel imager ChemiDoc was obtained from Bio-Rad, USA.

Murine CA model and treatment

Murine model of CA was constructed as previously described (Hosaka *et al*., 2014). Briefly, mice were anesthetized by injecting intraperitoneally 2% pentobarbital sodium (40 mg/kg). After laparotomy, bilateral renal arteries were separated under a microscope and the posterior branches were ligated. One week later, the left common carotid artery was exposed and ligated. The animals were randomly divided into three groups (n=6) to receive the surgery and diet containing 8% sodium chloride (model group), additional 30 mg/kg/d RES (RES group) selected from the low dose group in the previous work (Sebori *et al*., 2018) or sham operation (after laparotomy, the bilateral renal artery was not ligated) and fed with regular diet. RES was mixed with the diet that was orally given to the mice in the morning and mice were monitored to ensure the RES-containing diet was completely consumed before being given an additional diet. The feeding was from 9 weeks to 23 weeks of age. Sodium chloride was used to induce hypertension to enhance the incidence of lesions (Nagata *et al*., 1980). Four weeks after the treatment, mice were euthanized by CO_2 asphyxiation supplied at a flow rate of 20% of the cage volume per minute (5L/min). The brain was taken out by craniotomy, the basilar artery ring and its main branches were isolated under an anatomical microscope for subsequent analysis.

Blood pressure measurement

Blood pressure was measured in the tail of the mice using volume pressure recording (VPR) sensor technology with the noninvasive blood pressure monitor (CODA® High Throughput System, Kent Scientific, Torrington, CT, USA). Five readings were made for each mouse once a day. Blood pressures were reported as average mean arterial pressure (MAP) ± standard error of the mean (S.E.M.).

Hematoxylin and eosin (HE) staining

Aortic vessels were isolated, fixed, embedded in paraffin, and sectioned to 4 μm thick. The slices were routinely dewaxed, hydrated, and stained with HE as described and examined under a light microscope.

qRT-PCR

Total RNA was extracted from the vascular tissue using Trizol reagents according to the supplier's instructions. 200 ng of RNA was reversely transcribed to cDNA using the cDNA synthesis kit according to the supplier's instructions. The reaction was performed on a 96 thermal cycler with the following profile: 10 min at 25°C, 2 h at 37°C, and 5 min at 85°C. The synthesized cDNA was used as a template for qRT-PCR performed on ABI 7500 qRT-PCR instrument using TaqMan gene expression assay probes (Applied Biosystems). The PCR was carried out in a total volume of 10 μl containing 1.5 μl of diluted cDNA, 10 μl of TaqMan Gene Expression Master Mix, and 1 μl of each fluorescence TaqMan probe. The cycling conditions were 52ºC for 2 min, 94ºC for 10 min followed by 35 cycles, each one consisting of 15 s at 94ºC and 1 min at 60ºC. Samples were run in triplicate. Relative expression was calculated by using the comparative Ct method to obtain the fold change value ($2^{-\Delta\Delta Ct}$) according to the previously described protocol (Livak & Schmittgen, 2001). GAPDH was used as a reference gene. The primer sequences for PCR were

NF-κB:
- forward 5'-ACGATCTGTTCCCTCATC,
- reverse 5'-TGCTTCCCAGGAATA;
- forward 5'-GCAGCACTACTTCTTGACCACC,
- reverse 5'-TCTGCTCCTGAGCATTGACGTC;

MMP-2:
- forward 5'-CTGATAACCTGGATGCAGTCGT,
- reverse 5'-CCAGCCAGTCCGATTTGA; MMP-9:
- forward 5'-TTCAAGGACGTCGGTATT,
- reverse 5'-CTCGAGCCTAGACCCAACTTA;

GAPDH:
- forward 5'-AAGAAGGTGTGAAGGC,
- reverse 5'-TCCACCCTGTTGCTGTA.

Western blot analysis

After different treatments, the cerebral artery tissues were isolated and lysed with RIPA buffer containing protease and phosphatase inhibitors and quantitated using a BAC kit according to the manufacturer's instructions. 50 μg proteins were applied to 12% polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and then detected by incubating with the proper primary and secondary antibodies before visualization with a chemiluminescence kit. The intensity of blot signals was quantitated using an ultrasensitive chemiluminescence imaging system (ChemiDocXRS$^+$).

Statistical analyses

The normality of distribution of continuous variables was tested by a one-sample Kolmogorov-Smirnov test and the homogeneity of variance was assessed using Levene's test. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., USA). All experiments were repeated at least three times and performed in triplicate. Means between the groups were

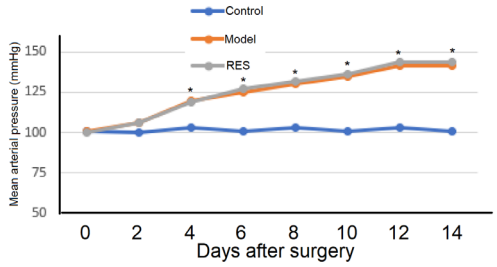

Figure 1. Mean arterial pressure of mice (N=6) after modelling surgery and RES treatment.
Data are presented as mean ±S.E.M. *denotes $P<0.05$ compared to control.

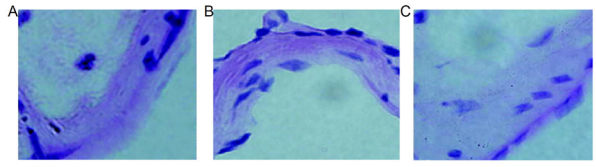

Figure 2. Hematoxylin and eosin (HE) staining of the anterior cerebral artery/olfactory artery bifurcation of mice after treatment with RES for I month.
(**A**) control mouse, (**B**) model, and (**C**) after RES treatment.

compared using the student's *t*-test. The frequencies of categorical variables were compared using Fisher's exact test. A *P*-value ≤0.05 was considered statistically significant.

RESULTS

RES inhibited the CA formation

The MAP of mice began to increase one day after the operation and was significantly higher than the control 4th day after the operation ($P<0.05$). It reached plateau 2 weeks after the operation (Fig. 1). Compared with control, MAPs were significantly higher in model (144.64±6.65 mmHg) and RES (145.86±9.14 mmHg) groups ($P<0.05$). However, MAPs between the model and RES were not different significantly ($P>0.05$). 4 weeks after the treatment, mice were sacrificed to examine the CA formation. Histological examinations showed that aneurysms of various sizes were formed in 4 out of 6 mice after modelling, while only one animal had a small aneurysm and a few swellings of the arterial wall in the bifurcation of the artery (Fig. 2 and Table 1). The incidence of CA in the resveratrol group was significantly lower than that in the model group (16.6% *vs* 66.7%, $P<0.01$). In the control group, the basilar artery ring was normal. Measurements showed that the size of CA was significantly reduced in the RES group as compared to the model (Table 1; $P<0.05$), while the arterial wall was thicker in the RES group as compared to the model (Table 1; $P<0.05$).

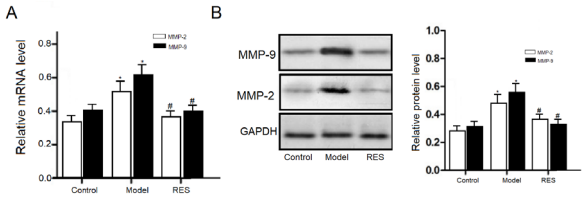

Figure 3. mRNA and protein expression of MMP-2 and MMP-9 in mice (N=6) 4 weeks after modelling surgery and RES treatment.
Data are presented as mean ±S.D. of three independent experiments. *and #denotes $P<0.05$ compared to control and model groups, respectively.

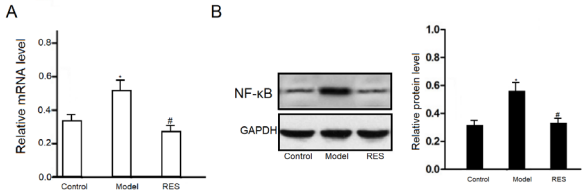

Figure 4. mRNA and protein expression of NF-κB in mice (N = 6) 4 weeks after modelling surgery and RES treatment.
Data are presented as mean ±S.D. of three independent experiments. *and #denotes $P<0.05$ compared to control and model groups, respectively.

RES decreased the inflammatory response of the CA wall

Compared with the model group, there were fewer infiltrated macrophages in the artery wall in the RES group (12.56±8.02 *vs* 7.05±1.69, $P<0.05$). In addition, MMP-2 and MMP-9 expression at both mRNA and protein levels were significantly up-regulated in model groups as compared to the control and significantly down-regulated in the RES group as compared with the model (Fig. 3A and B, $P<0.05$).

RES down-regulated NF-κB expression

NF-κB is a key transcriptional factor regulating inflammatory response. Compared with the control group, NF-κB expression at mRNA and protein levels was significantly elevated in the model group, but significantly downregulated after RES treatment (Fig. 4A and B, $P<0.05$) as compared with model group.

DISCUSSION

The results obtained in the study showed that RES could attenuate the CA formation in mice after induced hypertension, reduce the inflammatory response, and downregulate the expression of MMPs and NF-κB. These findings indicate the RES might be further ex-

Table 1. Formation of cerebral aneurysm and macrophage infiltration in mice following resveratrol treatment

Group	No. nice examined	No. cerebral aneurysm	Diameter of cerebral aneurysm (μm)	Thickness of artery wall (μm)	No. infiltrated macrophages
Control	6	0	0	21.6±2.3	2.05±0.69
Model	6	4	79.6±10.3	16.7±1.3*	12.56±8.02*
Resveratrol	6	1#	23.1±2.3#	±2.0	7.05±1.69#

*and #denote $P<0.05$ as compared with control and model, respectively.

plored as a therapeutic agent for preventing and treating the disease.

CA is a polygenic disease. In recent years, the role of MMPs in the CA formation has been increasingly addressed, although the mechanisms have not been fully elucidated (Kaneko *et al*., 2011b). MMPs are calcium-dependent and zinc-containing zinc- and calcium-dependent endopeptidases that are capable of degrading ECM proteins, such as collagen and non-collagen glycoprotein and elastin in extracellular matrix (Liu *et al*., 2006). They are mainly secreted by infiltrated macrophages and play an important role in tissue remodeling (Ota *et al*., 2009). Among MMPs, MMP-2 and MMP-9 are shown to have a close relationship with aortic aneurysm (Huffman *et al*., 2000; Rabkin, 2017), which is characterized by the loss of smooth muscle cells in the aortic media and the destruction of ECM. Studies confirmed that the activity of MMPs such as MMP-1, -2, -3, -9, -12, and -13 is elevated in abdominal aortic aneurysm and thoracic aortic aneurysm (Rabkin, 2017). The activity is higher in raptured CA (Qi *et al*., 2004) as compared with unruptured CA (Qi *et al*., 2004. In patients with CA, the mRNA levels of MMP-2 and MMP-9 in serum and aneurysm are significantly elevated, particularly in patients with ruptured CA, suggesting these proteinases contribute to the evolution of CA.

MMP-2 and MMP-9 are downstream molecules of the NF-κB signaling pathway, which regulates the expression of inflammatory genes, such as interleukin-1 (IL-1), induced nitric oxide synthase (iNOS), and MMPs and is regarded as a key mediator of CA formation. It is activated in cerebral arterial walls in the early stage of aneurysm formation, leading to up-regulated expression of downstream genes (Aoki *et al*., 2007b). A large number of studies have shown that chronic inflammation plays an important role in the formation and progression of CA, including the expression of IL-1β in vascular media at an early stage of aneurysmal formation (Hosaka & Hoh, 2014; Moriwaki *et al*., 2006). NF-κB up-regulates MCP-1 expression to mediate the macrophage recruitment, which is an important process in the inflammatory response. IL-1β and iNOS can induce the apoptosis of vascular smooth muscle cells, resulting in damage to the vascular endothelium and the destruction of the internal elastic lamina (Moriwaki *et al*., 2006). MMP-2 and MMP-9 participate in the vascular remodelling after the degradation and destruction of collagen, while a selective inhibitor for MMP-2, -9, and -12, tolylsam, reduced the advanced aneurysms (Aoki *et al*., 2007c). Because of its important role in CA, endothelial NF-κB is proposed to be a possible therapeutic target for CA (Saito *et al*., 2013).

In this study, we found that RES reduces the number and size of CA as compared with the model. The vascular wall was relatively normal and there were fewer macrophages infiltrated in the aneurysm wall after RES treatment. As shown in this study, NF-κB and MMP-2 and MMP-3 were up-regulated in the mice with induced hypertension, which was observed in both the model and resveratrol group likely as a result of high sodium diet (Nagata *et al*., 1980), and down-regulated after RES treatment, thereby reducing the infiltration of macrophages and inflammation response. Comparable results were observed in the rat model of myocardial infarction after treatment with tanshinone II A (Ren *et al*., 2013). NF-κB is a transcriptional factor that regulates the expression of a series of inflammation-related genes. Chronic inflammatory reactions such as macrophage infiltration into the arterial wall, release of cytokines, leukocytes, complement, immunoglobulins, and other humoral mediators could activate NF-κB and subsequently MMPs expression, promoting the formation and evolution of CA (Chalouhi *et al*., 2012; Starke *et al*., 2013). It is reported that the deficiency of the NF-κB p50 subunit or use of NF-κB oligonucleotide could inhibit the NF-κB activity, thereby suppressing the formation and evolution of CA (Aoki *et al*., 2007c). In addition, several studies have shown that resveratrol inhibits the NF-κB signaling pathway, leading to decreased NF-κB (S *et al*., 2020; Shang *et al*., 2019a; Yi *et al*., 2020). Inhibition of NF-kB signaling by resveratrol was also observed due to inhibiting p65 and IkB kinase (Ren *et al*., 2013). Our results are consistent with these data.

RES is one of the most important anti-microbial substances synthesized in plants when they are attacked by pathogens or a harsh environment (Vestergaard & Ingmer, 2019). It is shown to have a variety of biological functions, such as antioxidant activity, antitumor, and anti-inflammatory activity (Galiniak *et al*., 2019; Kang *et al*., 2009; Kumar & Sharma, 2010). Since RES has low water-solubility, in this study, the animals were administered with diet mixing with RES, instead of intraperitoneal injection, which would need to use organic solvents such as dimethyl sulfoxide to dissolve RES (Shang *et al*., 2019b), which could generate unknown side effect. Studies show that RES can inhibit the activation of NF-κB by phosphorylated IκB and LPS, leading to the suppression of downstream gene expression and the whole inflammatory response (Oh *et al*., 2009). Kaneko and others (Kaneko *et al*., 2011a) showed that RES inhibits the activity of many inflammatory factors in the vascular wall, such as TN-α, CD68, VEGF, P47, MMP2, and MMP9, and enhances neovascularization, thus preventing the formation and progression of aneurysm expansion. Palmieri and others (Palmieri *et al*., 2011b) also successfully prevented the early growth of abdominal aortic aneurysm in rats with RES by inhibiting the systemic and local inflammatory process in the vascular wall to avoid the degradation of the arterial wall. Taken together, it is clear that RES can to inhibit the elevation of NF-κB, MM-2, and MMP-9 expression and reduce the inflammatory response, leading to the prevention of CA formation and progression.

There are limitations to this study. It is an animal study and the results obtained need to be validated in other large animals or humans. The dose-response relationship needs to be studied to optimize RES administration and RES derivatives might be explored for better outcomes. Since RES was mixed into the diet, its bioavailability was not investigated and needs to be addressed in the future. In addition, the mice were not synchronized for their hormonal cycle, which might generate variability in their ability to produce an aneurysm.

CONCLUSION

Our study demonstrates that RES attenuates the formation and evolution of CA in mice after induced hypertension and this is likely mediated via downregulating the NF-κB pathway and reducing the inflammatory response. Further works with large animals and humans are needed to validate these findings and to explore RES as a therapeutic agent for CA.

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